IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John P. Atkinson

Serial No: 07/984,247 Art Unit: 1814

Filing date: November 30, 1992 Examiner: S.Walsh

For: Recombinantly Produced Human Membrane Cofactor Protein (MCP)

Commissioner of Patents
and Trademarks
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. §1.132

Sir:

I, Stephen Ip, hereby declare that:

1. I am the President and Chief Operating Officer at CytoMed, Inc., 840 Memorial Drive, Cambridge, MA 02139. CytoMed, Inc. is the licensee of the above-identified patent application. It also has an ongoing program to develop a MCP-based pharmaceutical.

2. Recombinant MCP was prepared and quantitated as follows.

Soluble MCP Purification

The Chinese hamster ovary (CHO) cell line producing soluble MCP was obtained from the laboratory of Dr. John Atkinson at Washington University. The cell line were frozen in liquid nitrogen. The frozen cell clone (MCP 3-6) was thawed in a

Corning T75 container with 20 mL of growth medium containing complete GMEM (Glasglow's Minimum Essential Medium) with 10% fetal bovine serum. After 24 hours in the 37° C incubator, 100 μM of MSX (methionine sulfoximine) were added. The culture medium was changed every several days until cell culture was 90% confluent.

For production of soluble MCP, cell culture production medium was collected after a 7-day incubation of medium in a 90% confluent cell culture containing CHO-1 (Ventrex), and 2 mM sodium butyrate. Soluble MCP was purified from culture medium with an anti-MCP monoclonal antibody column. The monoclonal antibody affinity column was prepared by coupling GB24 to Pierce Carbolink resin according to the manufacturer's specifications. CHO-1 production media containing soluble MCP was passed over the column, and washed with 10 mM sodium phosphate buffer pH 7.4 containing 150 mM NaCl. Bound protein was eluted with 0.1 M glycine-HCl buffer at pH 2.5.

Quantitation of sMCP by ELISA

An antibody capture ELISA assay was used to quantitate sMCP in cell culture supernatants. The anti-MCP monoclonal antibody GB24 was used as the capture antibody. ELISA plates were coated with 100 μl per well of a 1 μg/ml solution of GB24 in 0.1 M sodium bicarbonate buffer pH 9 overnight at 4°C. After washing, the plates were blocked with a solution of 1% BSA, 0.1%

NP-40 in PBS for 2 h at room temperature. For a standard curve, purified sMCP, the concentration of which had been previously been determined by protein assay, was added (50 µl/well in duplicate wells) in successive three-fold dilutions in blocking buffer at final concentrations ranging from approximately 200 ng/ml to 0.09 ng/ml. Supernatants containing sMCP of unknown concentration were diluted 1:10 in blocking buffer and in successive three-fold dilutions, then added to the plates. The plate was incubated for 1 h at 37°C and then washed with PBS. A peroxidase-conjugated secondary antibody which does not cross-compete with GB24 (antibody TRA-2-10) was added (50 µl/well of a 1:1000 dilution in blocking buffer) and incubated for 1 h at 37°C. After washing 100 µl/well of substrate (tetramethyl-benzidine) was added, and the peroxidase-catalyzed reaction was stopped with 50 µl of 2 N sulfuric acid. The plates were read at 450 nm. The concentration of sMCP in the culture supernatant was calculated by determining the concentration of the diluted sample where the Abs_{450} crosses the standard curve, and multiplying by the dilution factor.

3. The following studies were conducted to demonstrate that:

a. Soluble MCP protein (sMCP) can be generated by recombinant methods, in a pure form and large quantity.

b. Soluble MCP purified from recombinantly produced CHO cells has biological activities. These include:

i. Inhibition of complement activities of C3 convertase, as inhibition of the generation of C3a and Co-factor activity.

ii. Inhibition of complement activities of C5 convertase, as inhibition of the genration of C5a.

iii. Binding to C3b as determined by binding to iC3 and iC4.

iv. Inhibition of complement activity as demonstrated by its ability to inhibit sheep red blood cell hemolysis.

v. Inhibition of complement damages as demonstrated in a rat animal model of tissue injury in a passive reverse arthus reaction.

4. **Cofactor Activity of sMCP.**

The cofactor activity of SMCP was determined by the ability to enhance Factor I-mediated cleavage of iC3 or iC4. First, purified C3 and C4 (Diamedix) were converted to iC3 and iC4 (the internal thioester bond hydrolyzed) by either of two methods: (1) ten cycles of freeze/thaw, or (2) treatment with 4.0 M potassium bromide for 1 h at 37°C. The concentrations of iC3 and iC4 were adjusted to 0.5 mg/ml by dilution in dilution

buffer (0.5% NP-40 in 1:6 diluted PBS). Factor I (Quidel) solution was also prepared in dilution buffer at 66 μg/ml.

The assay was performed as follows: in a microcentrifuge tube, iC3 or iC4 (8 μl) were mixed with Factor I (2 μl) and 6 μl of either buffer or sMCP solution. The mixture was incubated for 1 h at 37°C. To stop the reaction, an equal volume of SDS sample buffer was added and the sample boiled for 5 min. The sample was run on a 10% polyacrylamide SDS gel, stained with Coomassie Blue and destained. To quantitate cleavage of the iC3 alpha chain, the gel was scanned on a gel scanner and the relative amounts of the cleaved and uncleaved chain determined by absorbance. Cofactor activity was directly proportional to alpha chain cleavage.

Attachment A is a graph of the percentage of alpha chain reduction versus concentration of sMCP (pM) showing that sMCP in the presence of 1.31 μM iC3 and 0.091 Factor I has cofactor activity.

4. **Effect of sMCP on sheep RBC Hemolysis.**

MCP activity was determined by its ability to inhibit the complement-mediated lysis of sensitized (rabbit IgG-coated) sheep red blood cells (sRBC). First, human serum was titrated for its ability to lyse sRBC. A dilution of serum was used at which 80-90% hemolysis occurs after 30 min incubation at 37°C. Once this dilution was determined, the assay was performed with

MCP as follows: sRBC (100 µl) and diluted serum (50 µl) were added together with varying concentrations of purified sMCP (100 µl). After incubation at 37°C for 30 min, the tubes were centrifuged for 15 sec. The supernatants were removed and read at 405 nm. Inhibition by sMCP was determined by the decrease in OD 405 in the sMCP-containing samples compared to the positive control (the sample not containing sMCP).

Attachment B is a graph of the percent inhibition of sensitized sheep red blood cell hemolysis by sMCP (µM), showing greater than 50% inhibition at concentrations of about 4 µM.

Attachment C is a graph of the percent inhibition of sensitized sheep red blood cell hemolysis in rat serum by sMCP, showing that 10 µg sMCP yields 25% inhibition and 20 µg sMCP yields 50% inhibition.

5. **iC3 and iC4 Binding Activity of sMCP.**

The ability of sMCP to bind iC3 and iC4 was determined by its removal from solution after passage through Sepharose-immobilized ligand. First, iC3 and iC4 (Diamedix) were separately conjugated to Sepharose by the CNBr activation method. Next, the initial concentration of either a solution of purified sMCP or a cell culture supernatant was determined by ELISA. The solution should be assayed for ligand binding activity at a concentration resulting in 80-90% saturation by ELISA. Once this solution of sMCP was prepared, 100 µl was mixed with 100 µl of

iC3- or iC4-Sepharose slurry and incubated for 1 h at 37°C. A negative control was also run, usually with BSA-conjugated Sepharose. After incubation, the slurry was centrifuged and the supernatant assayed again for sMCP concentration by ELISA. The difference in sMCP content before and after binding to the affinity resin represents the amount bound to the column. This was expressed as a percentage of total sMCP.

Attachment D is a graph of the percent of iC3 degradation versus concentration of sMCP (pM) showing iC3 cofactor activity of sMCP.

Attachment E is a graph of the percent binding of iC3 and iC4 of sMCP, showing greater than 70% binding of iC3 and 60% binding of iC4.

7. **Effect of sMCP on C5a Generation Zymosan Activation of NHS.**

Since MCP inactivates C3b by converting it to C3bi, it therefore inhibits the activities of C3 and C5 convertases. This activity can be demonstrated by inhibiting the generation of C3a and C5a. Complement activation *in vitro* can be accomplished by either the classical or alternative pathways. For the classical pathway, aggregated rabbit immunoglobulin at 10 mg/ml is used. For the alternative pathway, 10 mg/ml zymosan is used.

The assay was performed as follows: undiluted human serum (50 µl) and sMCP at various concentrations were mixed in a

total volume of 180 µl in Veronal buffer. Either aggregated IgG or zymosan was added (20 µl) and the samples incubated for 30 min at 37°C. EDTA solution (20 µl) was added to stop the reaction. The amount of C3a or C5a in the sample was determined with RIA kits from Amersham. Precipitating reagent (220 µl) was added to the sample and incubated for 15 min. After centrifugation, the supernatant was assayed for C3a or C5a by the RIA according to the manufacturer's protocol.

Attachment F is a graph of the percent inhibition versus concentration of sMCP (µM), showing the effect of sMCP on C5a generation.

8. **Reverse Passive Arthus Reaction Effect of sMCP.**

The *in vivo* activity of soluble MCP was tested with an inflammatory rat animal model using a reverse passive arthus reaction. 200-250 gm Sprague-Dewley rats were anesthetized with i.v. injection of 2 ml/kg of sodium pentobarbital solution containing Evan blue and 4 mg/ml of ovalbumin. 10 minutes after injection 40 µl of anti-ovalbumin or 40 µl anti-ovalbumin with 25 µl to 110 µl of soluble MCP were injected intradermally. After 2.5 hours of reaction, rats were sacrificed, skin removed, and evan blue dye were extracted with 3 ml of extraction solution containing 2 ml of acetone and 1 ml of 0.5% aqueous sodium sulfate. After centrifuging the extraction solution at 1000 x g

for 10 minutes, the amount of blue dye was determined at 620 nm with a spectrophotometer.

Attachment G is a graph of the percent of control versus sMCP concentration (μM) showing the effectiveness *in vivo* of sMCP in inhibiting an inflammatory reactions, at concentrations of 3.63, 7.27, and 16.00 μM.

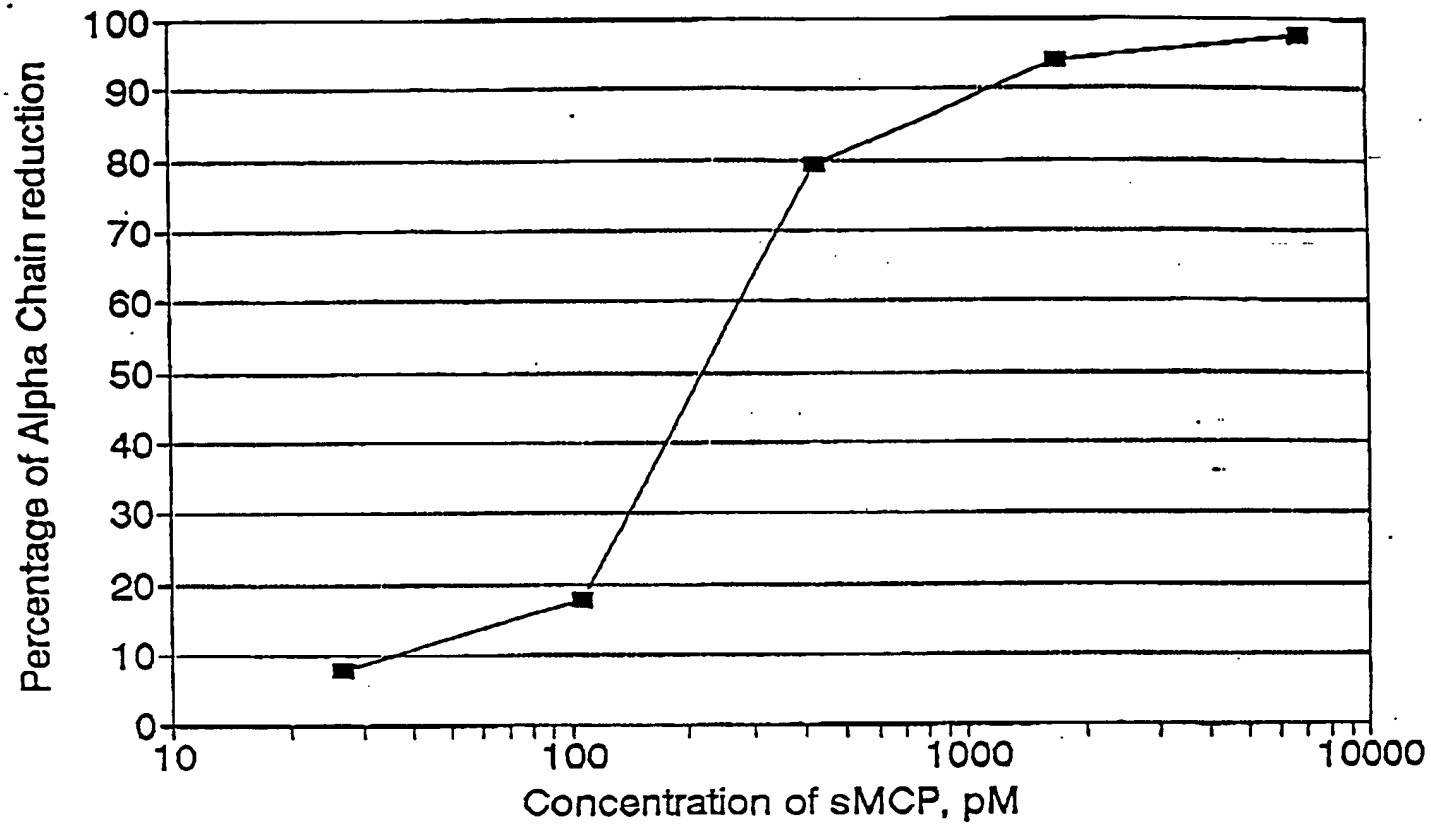
9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: July 12/93 Stephen H Ip

Stephen Ip

Cofactor Activity of sMCP

1.31 uM iC3 & 0.091 uM Factor I



EXHIBIT

A

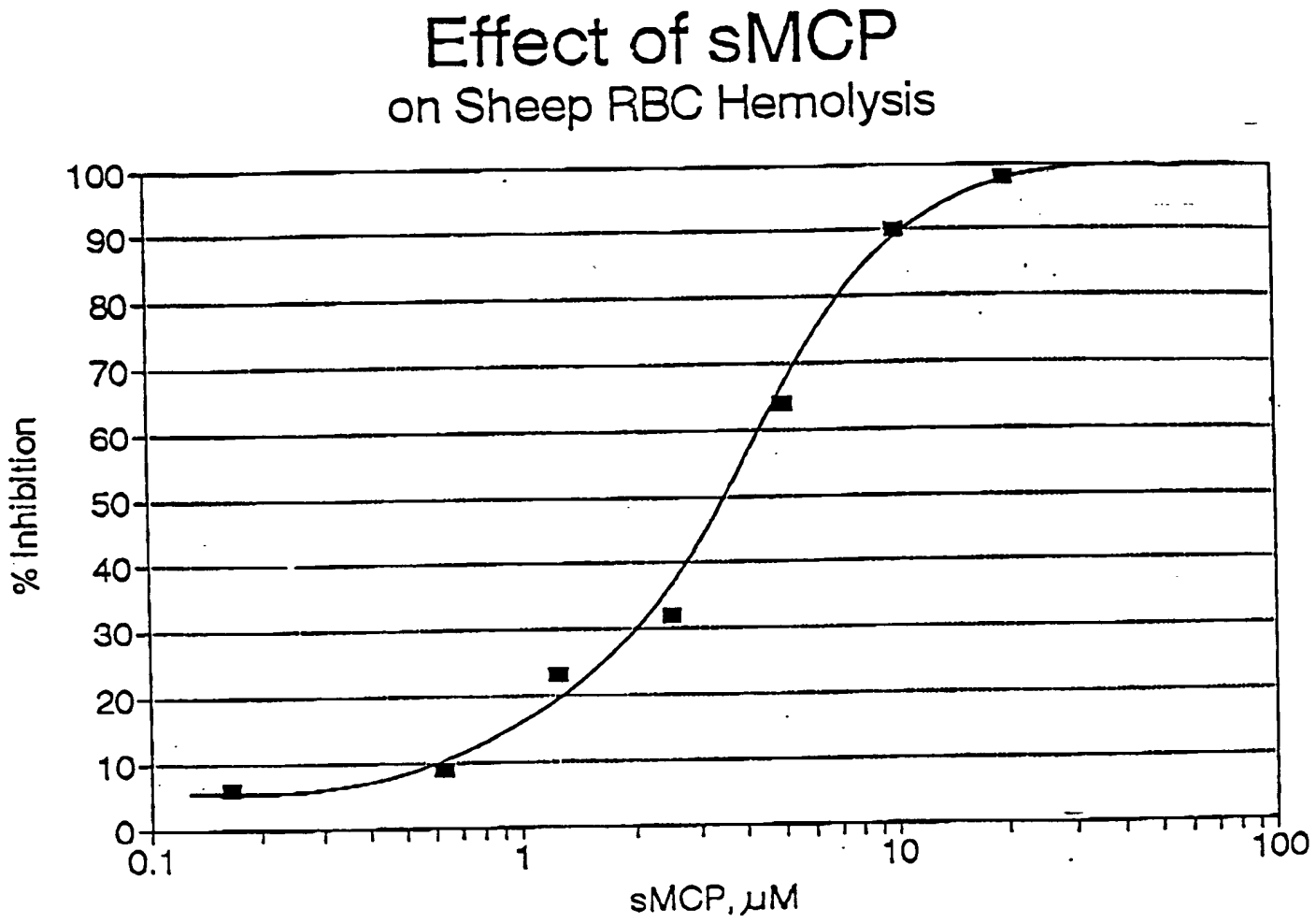


EXHIBIT
B

Inhibition of sensitized sheep red blood cell hemolysis by sMCP.

Effect of sMCP on sRBC Lysis
Rat Serum

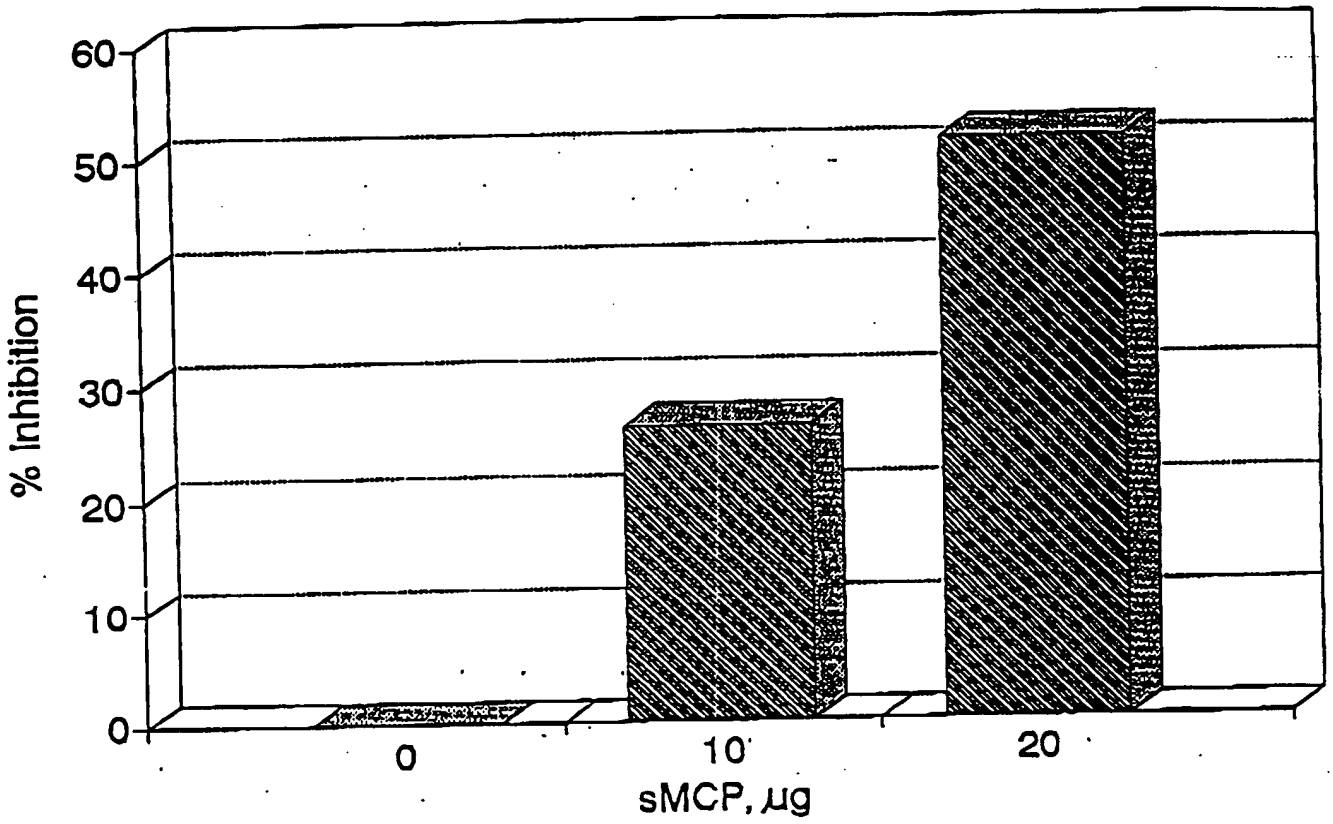
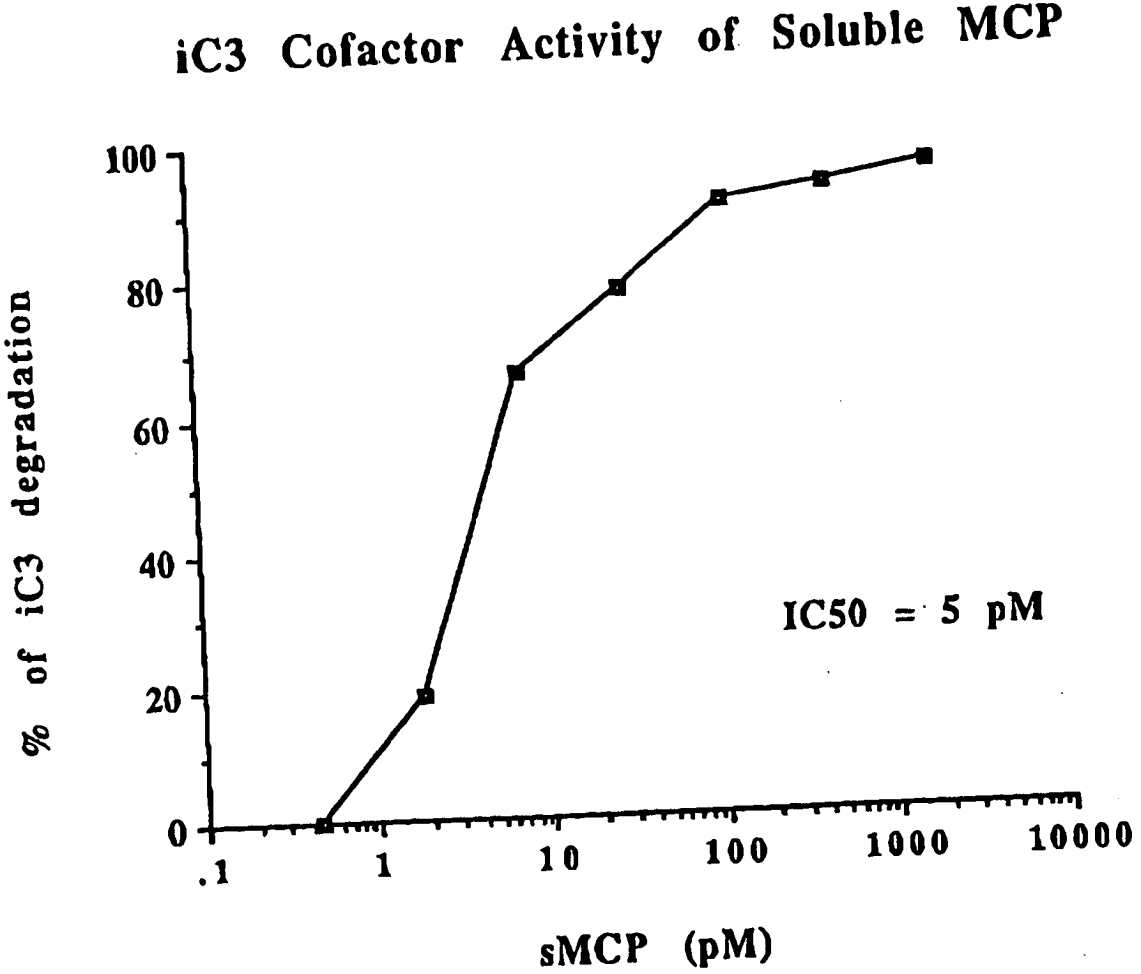


EXHIBIT
C

Inhibition of sensitized sheep red blood cell hemolysis in rat serum by sMCP.


iC3 Cofactor Activity of Soluble MCP
% of iC3 degradation
100
80
60
40
20
0
.1
1
10
100
1000
10000
IC50 = 5 pM
sMCP (pM)

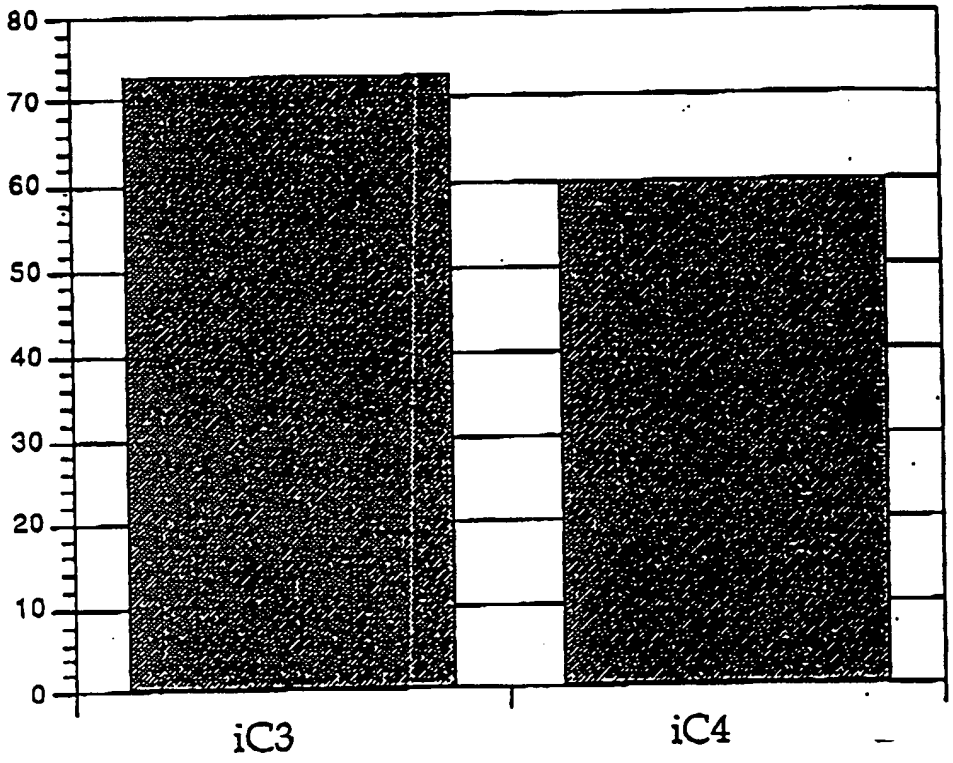
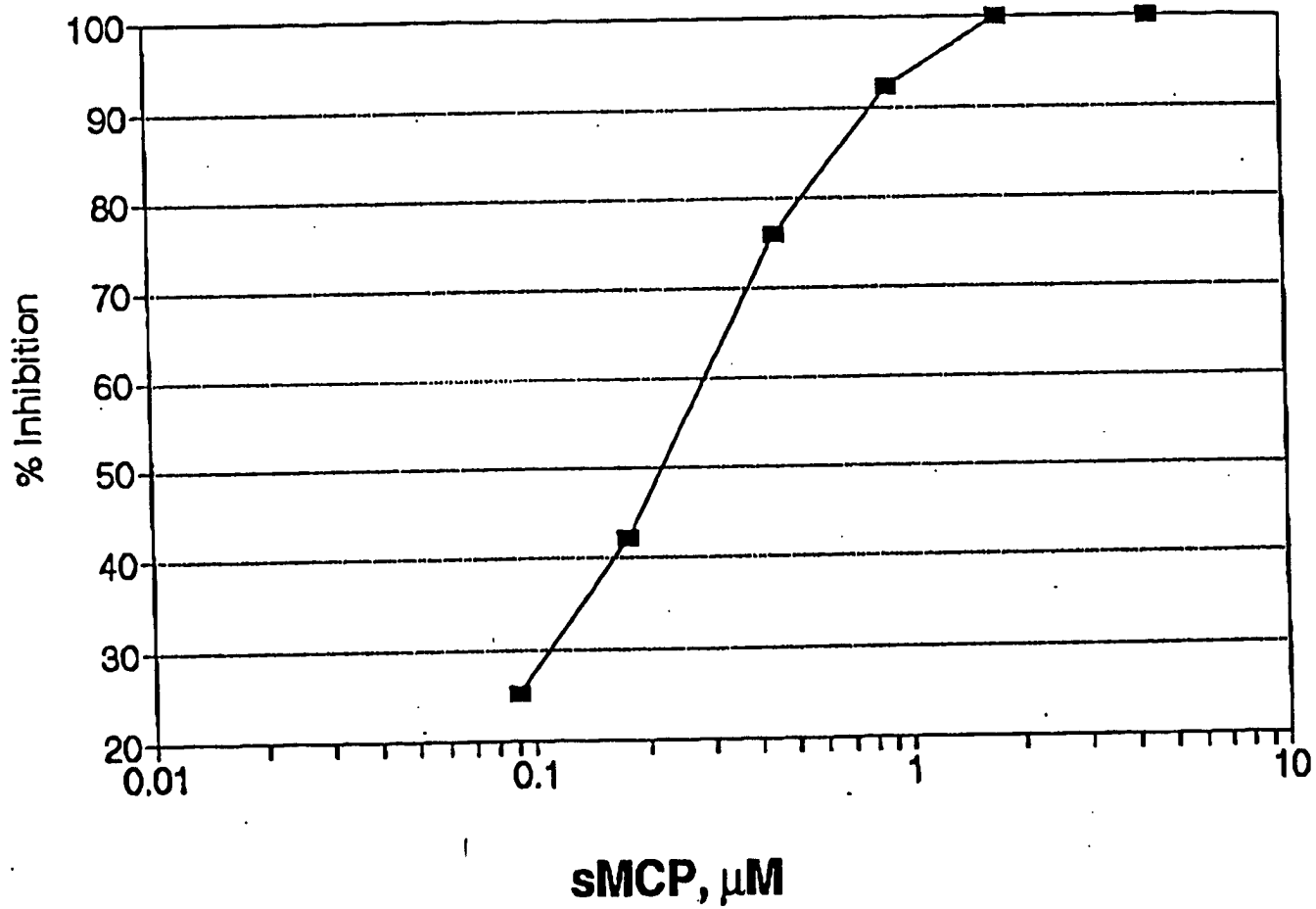


EXHIBIT
E

iC3 and iC4 binding activity of sMCP.

Effect of sMCP on C5a Generation Zymosan Activation of NHS



Reverse Passive Arthus Reaction
Effect of sMCP

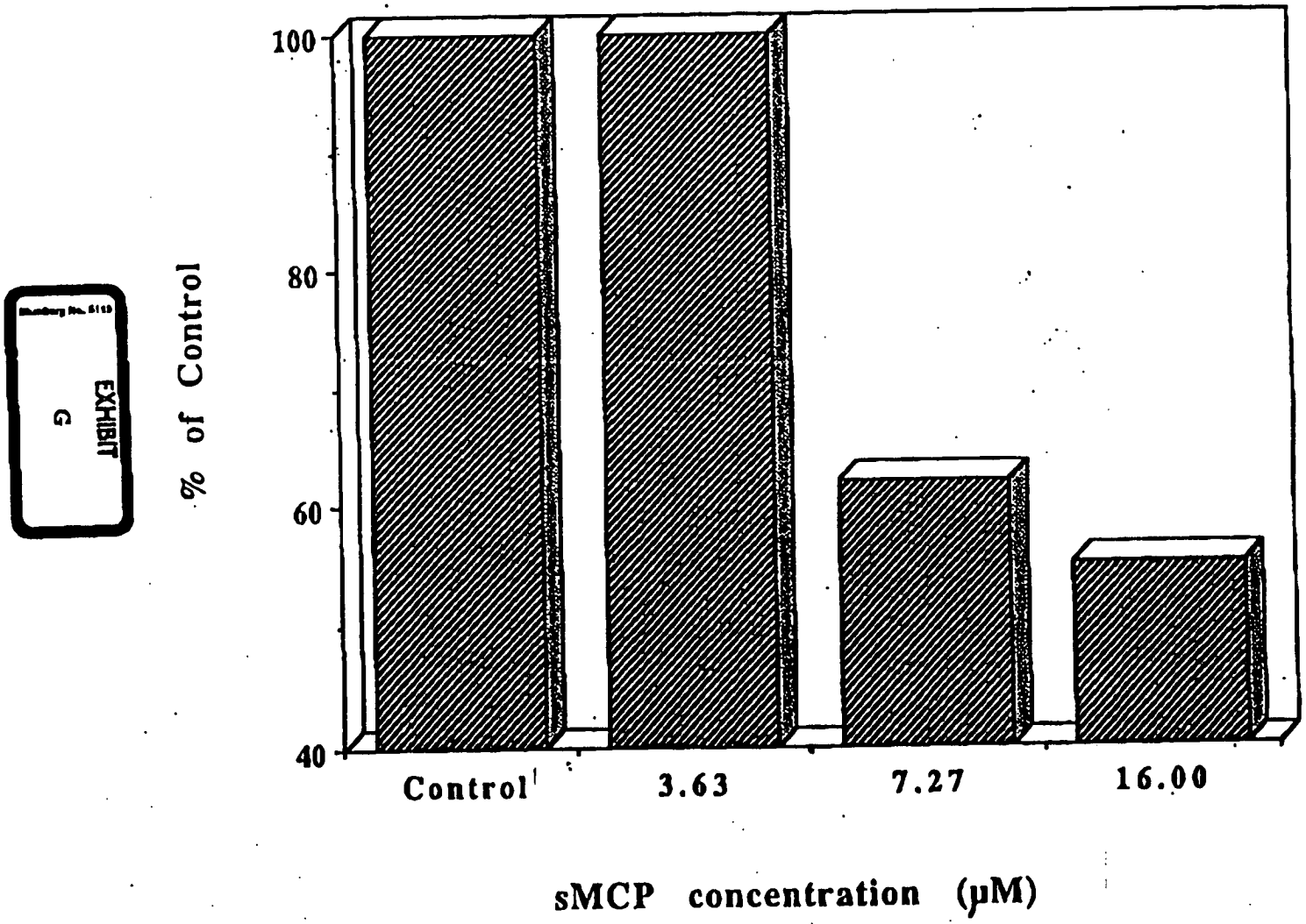


EXHIBIT G